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Increasing permeability of phospholipid bilayer membranes to alanine with synthetic α-aminophosphonate carriers

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Abstract—A series of aminophosphonates was synthesized, and their ability to carry alanine, a model hydrophilic molecule, across phospholipid bilayer membranes was evaluated. Aminophosphonates facilitate the membrane transport at moderate rates, which make them a suitable platform for the design of carriers for continuous drug release devices.

One of the most important challenges in designing liposome based drug release systems is the precise control of transport kinetics across bilayer boundaries.[1,2] Encapsulated drugs should remain within liposomes during storage, and release should only begin after the administration. In an ideal system, a triggering event will initiate continuous release with well-controlled rate. Synthetic carriers of polar organic molecules through lipid bilayers can potentially provide an elegant way to achieve this goal. Insertion of carriers into bilayers of drug-containing liposomes immediately prior to administration would trigger the release. Transport kinetics can be regulated by varying the concentration of carriers in the bilayer.

Previously, a relatively small number of studies focused on synthetic carriers for the transport of organic molecules across bilayer lipid membranes compared to well explored areas of artificial ion channels[3,4] and transport in supported liquid membranes.[5–8] In the recent years, however, the field has been gaining considerable interest. Several papers described synthetic carriers for carbohydrates,[9,10] nucleosides,[11] small peptides,[12] and other molecules.[13,14] Among the most recent beautiful examples is the transport of oligonucleotides guided by umbrella carriers in the innovative 'needle and thread concept'.[15] Sunamoto et al. reported on the transport of phenylalanine using a photoresponsive carrier.[16]

Traditionally, high selectivity and fast transport rates were viewed as desirable albeit challenging. These characteristics may not be necessary in the design of carriers suitable for continuous drug release. This application requires slow release of a single component over a long period of time, measured between hours and weeks, and neither selectivity nor fast transport is critical. In fact, slow carriers are likely to have an advantage of inherently low cytotoxicity, an important safety consideration in the case a carrier molecule separates from a liposome and inserts into a cellular membrane. Small size of carriers is likely to be beneficial for rapid incorporation into the liposomal bilayer.

Considering the above, we decided to synthesize and evaluate a series of α-aminophosphonates as carriers of hydrophilic organic molecules across phospholipid membranes. We used alanine, an amino acid, as a model hydrophilic bifunctional molecule for transport studies. In our view, aminophosphonates are excellent carrier platforms. They possess two binding sites, a hydrogen bond donor and a hydrogen bond acceptor suitable for two-point binding of hydrophilic molecules creating in the case of amino acids a complex with hydrophobic exterior.[17,18] They are readily prepared in a one-pot synthesis by the Kabachnik–Fields reaction from a primary amine, a phosphite, and a carbonyl compound.[19,20] Their hydrophilic–hydrophobic balance can be easily

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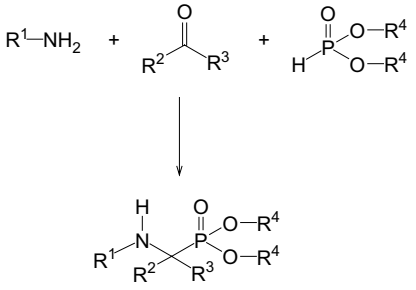
varied by using different substituents in the starting materials. In previous studies, aminophosphonates did not exhibit cytotoxicity or mutagenic properties.[21,22] Previously reported non-toxic concentrations as high as 15 mM support the feasibility of successful in vivo applications.[21,22] Base aminophosphonate platform can be further modified with substituents that provide steric interactions or additional binding of a substrate molecule. This can be utilized to adjust the selectivity and strength of binding of a wide range of substrates. Three-point interactions were shown effective in chiral recognition.[23]

The synthesis of the α-aminophosphonates was done by the Kabachnik–Fields reaction, a condensation between an amine, a carbonyl compound, and a phosphite (Scheme 1).[24]

By using aniline and benzylamine, acetone and cyclohexanone as carbonyl compounds and dimethylphosphite, dibutylphosphite, or bis-2-ethyhexylphosphite as phosphites, we synthesized a series of seven aminophosphonates. We selected substituents for achieving variations in size and hydrophobic–hydrophilic balance of products. Thus compound **3** is almost twice the size of compound **1**. Compounds **2** and **4** are similar in size but are different in structure. Products were isolated by column chromatography on silica gel using hexane/ethyl acetate (5:1) as eluent. The most prominent feature



1 $R^1 = C_6H_5$; $R^2 = R^3 = CH_3$; $R^4 = CH_3$
2 $R^1 = C_6H_5$; $R^2 = R^3 = CH_3$; $R^4 = C_4H_9$
3 $R^1 = C_6H_5$; $R^2 = R^3 = CH_3$; $R^4 = CH_2CH(CH_2CH_3)C_4H_9$
4 $R^1 = C_6H_5$; $R^2 + R^3 = (CH_2)_5$; $R^4 = CH_3$
5 $R^1 = C_6H_5$; $R^2 + R^3 = (CH_2)_5$; $R^4 = C_4H_9$
6 $R^1 = CH_2C_6H_5$; $R^2 = R^3 = CH_3$; $R^4 = CH_3$
7 $R^1 = CH_2C_6H_5$; $R^2 + R^3 = (CH_2)_5$; $R^4 = C_4H_9$

Scheme 1. Synthesis of α-aminophosphonates **1**–**7** from an amine (aniline or benzylamine), a carbonyl compound (acetone or cyclohexanone), and a phosphite (dimethyl phosphite, dibutyl phosphite, or bis-(2-ethylhexyl)-phosphite).

in ^{1}H NMR spectra that indicated the formation of the condensation product was the long-range coupling between phosphorus and hydrogens contributed by the carbonyl compound.

Transport properties of aminophosphonate carriers were evaluated by liposome efflux experiments coupled with enzymatic assays.[25] In these experiments, unilamellar liposomes loaded with alanine (300 mM) were prepared from dipalmitoyl phosphatidyl choline (DPPC) and cholesterol (3:1 molar ratio) by a standard literature protocol,[26] and unentrapped alanine was separated by size exclusion chromatography. The batch of alanine-loaded liposomes was separated into several samples, and identical amounts of carriers were introduced to bilayers with the help of a solvent vector, DMSO, so that each sample contained a different carrier with the same concentration. The rate of alanine efflux was determined from the conversion of alanine to pyruvate, catalyzed by Glutamic-Pyruvic Transaminase, and further to lactate, catalyzed by lactate dehydrogenase, which is accompanied by the oxidation of nicotinamide cofactor NADH to NAD^+. Disappearance of NADH was monitored by UV spectroscopy.[27]

The carrier mechanism of transport was supported by the lack of evidence of aminophosphonate induced membrane lysis. In this experiment, we prepared liposomes containing self-quenching fluorescent dye calcein. Calcein loses 98% of its fluorescence at concentrations above 100 mM.[28] In the lysis experiment, liposomes with calcein were mixed with aminophosphonate solution in DMSO and the fluorescence was monitored for 1 h, the amount of time comparable with the amino acid efflux experiments. No detectable change in emission was observed. In the end of this period, liposomes were treated with Triton X-100 solution that caused lysis[26] and an immediate huge increase of emission, indicative of dye dilution below self-quenching concentration due to release from liposomes. We conclude that the aminophosphonates did not cause bilayer membranes to become more permeable, as this would have been accompanied by a continuous increase of emission.

Data in Table 1 summarize average flow rates for alanine using different aminophosphonates. All carriers increase permeability of phospholipid bilayers to alanine. In control experiments, we observed no measurable enhancement of alanine transport when adding amines or phosphites to the bilayers. Flux was calculated in nmol/h × m^2 using volume and surface area of 100 nm liposomes, which was the average size confirmed by transmission electron microscopy. We chose these units of measurement to enable comparison with other studies. Thus, we calculated the photoresponsive carrier-as-

Table 1. Transport of alanine across lipid bilayers, average flux from three experiments

Carrier	None[a]	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[a]	**7**[a]
Flux, nmol/h × m^2	0.7 (±0.4)[b]	4.4 (±1.6)	3.1 (±1.0)	4.3 (±2.0)	7.2 (±1.8)	4.4 (±0.5)	7.8 (±2.3)	4.4 (±1.8)

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] Measured by a liposome efflux experiment coupled with alanine dehydrogenase assay.

sisted transport of phenylalanine to be 14 nmol/h × m^2 from the data reported by Sunamoto et al., which is comparable to the values we observed.[16] Since all carriers were always run together using the same batch of liposomes, relative transport rates offer a meaningful comparison. Compounds **4** and **6** exhibited fastest transport rates in the series, and compound **3** was the slowest carrier.

We observed no direct relationship between hydrophobicity and efficiency of aminophosphonate carrier. It is likely that optimum transport efficiency is the function of availability of the carrier at the bilayer boundary, hydrophobicity of the complex, and mobility of both complex and free carrier. We observed significant differences between carrier **2** and carriers **4** and **6** as well as carrier **5** and carriers **4** and **6**. This can open an opportunity for finding relationships between carrier structure and transport efficiency.

Based on transport rates measured in this study, 50% release of alanine, encapsulated in a 100-nm liposome with the concentration of 300 mM, over a period of 3 weeks would require 4–5 mol % of aminophosphonate carriers relative to membrane phospholipids. Such concentrations are attainable, and can be scaled down for even longer release. Further tuning of transport efficiency through additional binding by side-arm substituents may offer control of mass transfer kinetics in a wider range. Rapid progress in long-circulating liposomes makes artificial transporters an attractive component of the drug delivery systems.[29]

In summary, we synthesized a series of α-aminophosphonates and evaluated their transport properties. Aminophosphonates are capable of transporting alanine, a model hydrophilic compound, across bilayer lipid membranes at moderate rates. Due to ease of structural variations, α-aminophosphonates can be attractive platforms for building carriers for facilitating transmembrane transport and regulating permeability of long-circulating continuous release drug delivery devices.

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24. Reagents and conditions: **1**, **2**, **3**, **6**: amine (0.01 mol), phosphite (0.01 mol), acetone (5 ml), 56 °C, overnight; **4**, **5**, **7**: amine (0.01 mol), cyclohexanone (0.01 mol), phosphite (0.01 mol), toluene (3 ml), 80 °C, overnight. Solvent was evaporated, and the mixture was separated by column chromatography on silica gel with hexane/ethyl acetate (5:1) eluent. Yields and spectroscopic data for the α-aminophosphonates are as follows (^{1}H NMR, $CDCl_3$, 270 MHz):
*Compound **1**:* Yield 75%, 1.48 ppm (d, 6H, *J* = 15.6 Hz), 3.72 ppm (d, 6H, *J* = 10.4 Hz), 6.82 ppm (t, 1H, *J* = 7.4 Hz), 7.02 ppm (d, 2H, *J* = 1.2 Hz), 7.16 ppm (t, 2H, *J* = 20.0 Hz).
*Compound **2**:* Yield 68%, 0.9 ppm (t, 6H, *J* = 32.4 Hz), 1.36 ppm (m, 4H, *J* = 51.3 Hz), 1.46 ppm (d, 6H, *J* = 15.6 Hz), 1.56 ppm (m, 4H, *J* = 21.6 Hz), 4.09 ppm (m, 4H, *J* = 21.0 Hz), 6.82 ppm (t, 1H, *J* = 7.4 Hz), 7.02 ppm (d, 2H, *J* = 1.2 Hz), 7.16 ppm (t, 2H, *J* = 20.0 Hz).
*Compound **3**:* Yield 50%, 0.86 ppm (t, 12H, *J* = 54.0 Hz), 1.28 ppm (m, 10H, *J* = 27.0 Hz), 1.33 ppm (m, 4H, *J* = 13.5 Hz), 1.48 ppm (d, 6H, *J* = 15.6 Hz), 1.55 ppm (m, 2H, *J* = 35.1 Hz), 3.93 ppm (m, 4H, *J* = 21.0 Hz), 6.82 ppm (t, 1H, *J* = 7.4 Hz), 7.02 ppm (d, 2H, *J* = 1.2 Hz), 7.16 ppm (t, 2H, *J* = 20.0 Hz).
*Compound **4**:* Yield 61%, 1.26 ppm (m, 2H, *J* = 27 Hz), 1.51 ppm (m, 4H, *J* = 14 Hz), 1.80 ppm (m, 2H, *J* = 40.5 Hz), 2.2 ppm (m, 2H, *J* = 19 Hz), 3.68 ppm (d, 6H, *J* = 10.4 Hz), 6.82 ppm (t, 1H, *J* = 7.4 Hz), 7.02 ppm (d, 2H, *J* = 1.2 Hz), 7.16 ppm (t, 2H, *J* = 20.0 Hz).
*Compound **5**:* Yield 39%, 0.86 ppm (t, 6H, *J* = 13.5 Hz), 1.30 ppm (m, 6H, *J* = 35.1 Hz), 1.51 ppm (m, 4H, *J* = 27 Hz), 1.8 ppm (m, 2H, *J* = 35.1 Hz), 2.2 ppm (m, 2H, *J* = 21.6 Hz), 4.09 ppm (m, 4H, *J* = 21.0 Hz), 6.79 ppm (t, 1H, *J* = 7.4 Hz), 7.02 ppm (d, 2H, *J* = 1.2 Hz), 7.14 ppm (t, 2H,*J* = 20.0 Hz).
*Compound **6**:* Yield 59%, 1.44 ppm (d, 6H, *J* = 15.6 Hz),

3.83 ppm (d, 6H, J = 10.4 Hz), 3.96 ppm (s, 2H), 6.79 ppm (t, 1H, J = 7.4 Hz), 7.02 ppm (d, 2H, J = 1.2 Hz), 7.14 ppm (t, 2H, J = 20.0 Hz).
Compound 7: Yield 51%, 1.24 ppm (m, 2H, J = 14.3 Hz), 1.51 ppm (m, 4H, J = 14.2 Hz), 1.73 ppm (m, 2H, J = 27.2 Hz), 1.87 ppm (m, 2H, J = 16.2 Hz), 3.78 ppm (d, 6H, J = 10.4 Hz), 3.9 ppm (s, 2H) 6.79 ppm (t, 1H, J = 7.4 Hz), 7.02 ppm (d, 2H, J = 1.2 Hz), 7.14 ppm (t, 2H, J = 20.0 Hz).

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27. Kinetic data were collected on Agilent 8453 UV–vis spectrophotometer equipped with 8-cell cuvette holder maintained at 37 °C. In a typical experiment, samples were prepared by mixing 100 μL of liposome solution prepared from 0.03 mmol of dipalmitoyl phosphatidyl choline and 0.01 mmol of cholesterol in 1 ml of alanine solution (300 mM) in tris buffer (50 mM, pH 7.4) and separated from unentrapped alanine on Sephadex-25 column, 100 μL of solution containing enzymes GPT and LDH and NADH, 795 μL of buffer, and 5 μL of α-aminophosphonate solution in DMSO (100 mM). NADH concentration change was calculated from absorbance at 340 nm. Background absorbance change in the control sample containing DMSO but no carrier was subtracted from all measurements to single out the effect of aminophosphonate carriers on alanine transport. The enzymatic assay was calibrated with aliquots of alanine of known concentrations. In all cases, complete conversion of alanine was observed.
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